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EFFECTS OF SEVERAL STAINS AND FLUIDS ON STAINING AND VIABILITY --ETC(U)
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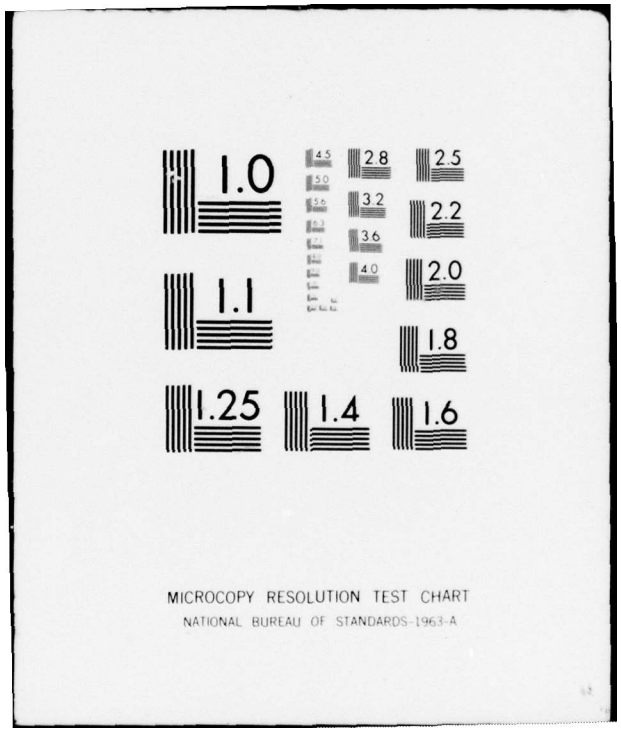
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TECHNICAL NOTE

EFFECTS OF SEVERAL STAINS AND FLUIDS ON STAINING AND VIABILITY OF SPORES OF PIRICULARIA ORYZAE

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January 1970

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ABSTRACT

Spores of Piricularia oryzae have been stained with a variety of stains. Methylene Blue provided the best staining properties for observation under white light; Acridine Orange and Rhodamine B produced the better fluorescing properties.

Spores of P. oryzae were also suspended in solutions of sodium chloride, "Acti-dione", "Tergitol 4", Rhodamine B, Phloxine B, Acridine Orange and Methylene Blue at various concentrations. Phloxine B inhibited spore germination completely. Cycloheximide, except at the 0.05% level, and "Tergitol 4", except at the 0.1% and 0.2% levels, inhibited spore germination also. "Tergitol 4" stimulated germination mildly at the lower concentrations employed. To a lesser degree Rhodamine B stimulated germination at 0.05%, 0.2% and 0.1%. Methylene Blue stimulated germination at all concentrations tested except at the 3.0% level. Likewise, NaCl and Acridine Orange stimulated at all concentrations tested.

Acridine Orange is recommended for staining spores to be counted for direct or automatic counting equipment. A study of persistency of P. oryzae in local soils under ambient conditions is suggested.

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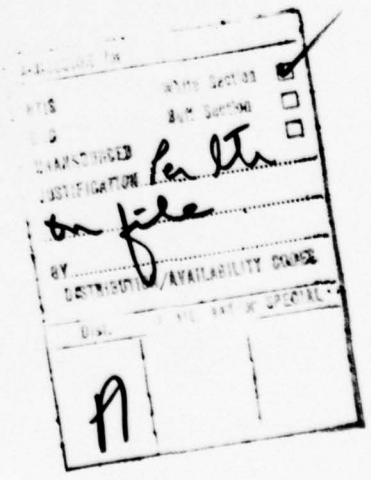

I. INTRODUCTION

In an earlier investigation[1*], live spores of Piricularia oryzae, causal agent of rice blast, were disseminated in a wind tunnel and collected with various samplers. Those samplers (Andersen and Gel Filters) on which P. oryzae exhibited growth, were easily assayed by direct colony count. Those samplers on which direct spore counting was required, were found to present difficulties due to the minute size of the spores, their lack of coloration, clumping and shriveling which probably resulted from impaction. Among such samplers were membrane filters and "u-shaped" rotorods. Even when spores were removed from the rotorod by the Tape Transfer Technique (DPG SOP #67) and placed on glass microslides for direct examination with transmitted light, assay was difficult and slow.

Staining or combination of a stain and an additive fluid that might aid in wetting the spore to improve staining uptake might provide a means by which these spores could be examined more easily and, therefore, more easily counted if such a direct method is required. This paper reports the effects of several stains and other fluids on the staining and viability of spores of P. oryzae.

* See "Literature Cited"

II. MATERIALS AND METHODS

Live spores of Piricularia oryzae were obtained from the U. S. Army Biological Laboratories, Ft. Detrick, Frederick, Maryland. All plated spores were plated in petri plates containing the following medium:

Corn Meal Agar (Difco)	19.0 g
Agar (Difco)	0.5 g
Distilled water	1000 ml
Stock Penicillin (5,000,000 units/10 ml H_2O)	10 ml
Stock Streptomycin (5 5/10 ml H_2O	1 ml

The antibiotics were added to the medium just prior to pouring into the petri plates.

TREATMENT #1: Direct Staining

Spores were suspended in sterile distilled water and a drop of the suspension was placed on a glass microslide. A drop of stain or other fluid (0.1%) was then placed on the moistened spores, permitted to stand for 5 minutes, covered with a glass coverslip and examined microscopically under white light. The following fluids and stains were utilized:

1. Distilled water
2. NaCl
3. Tergitol 4
4. Sudan IV
5. Lugol's Iodine
6. Iodine Potassium Iodide (IKI)
7. Lactophenol
8. Lactofuchsin
9. Melzer's Reagent
10. Lactophenol-Cotton Blue
11. Acid Fuchsin
12. Basic Fuchsin
13. Malachite Green
14. Nigrosin
15. Rose Bengal
16. Safranin
17. Acetothionine
18. Gentian Violet
19. Amman's Fluid
20. Methylene Blue
21. Phloxine B
22. Rhodamine B
23. Fluorescein Isothiocyanate
24. Acridine Orange

In addition, microslides of spores in distilled water and those stained with Rhodamine B, Fluorescein Isothiocyanate and Acridine Orange were examined under ultraviolet light for autofluorescence and fluorescent staining.

TREATMENT #2: Viability

Spores (0.1 g) were placed in 9.9 ml of varying concentrations of the

following stains and fluids and permitted to stand for thirty minutes; NaCl, Tergitol 4 (Union Carbide Co.), "Acti-dione" (Upjohn Co., brand of Cycloheximide), Phloxine B, Acridine Orange, Methylene Blue and Rhodamine B. Suspensions were plated in duplicate per standard methods (DPG SOP #8), incubated at $30^{\circ}C$ for 3-5 days and colonies of P. oryzae were counted and recorded. Serial dilutions (to 10^{-6}) were prepared of the several stains and fluids in the various concentrations were plated in duplicate, incubated and counted as noted above. Spores suspended in sterile distilled water were treated as were the above suspensions and served as the controls.

III. RESULTS AND DISCUSSION

In distilled water, NaCl and Tergitol 4, spores of P. oryzae exhibited a normal appearance (Fig. 1), although an occasional spore appeared to be somewhat aborted. Average measurements of these spores were 25.1 μ long x 8.5 μ wide (base) x 3.9 μ wide (apex).

In Sudan IV, Lugol's Iodine, IKI, Lactophenol, Lactofuchsin and Nigrosin little staining of spores occurred and plasmolysis (shrinkage of protoplasm due to water loss) and shriveled spores were frequent. This condition could have resulted from the fluid's action or their acidic nature. With Melzer's Reagent, Lactophenol-Cotton Blue, Acid Fuchsin, Basic Fuchsin, Malachite Green, Rose Bengal, Safranin, Acetothionine, Gentian Violet and Phloxine B, the cytoplasm only was stained from rather poorly to moderately well. Plasmolysis and shriveling was also frequent. The cytoplasm of basal and apical cells of spores stained well with Amman's Fluid, but the central cells remained clear. Both wall and cytoplasm were stained quite darkly by Methylene Blue (Fig. 3). No plasmolysis occurred and only an occasional aborted spore was observed in the latter two stains.

Cytoplasm and walls of spores were both stained by Rhodamine B and were readily visible in white light. Under ultraviolet light, however, only the walls exhibited fluorescence. Neither Fluorescein Isothiocyanate nor Acridine Orange (Fig. 2) exhibited staining of walls or cytoplasm in white light, but both stains provided fluorescence in spore walls under ultraviolet light. Bits of hyphal elements present in the spore mass, however, did fluoresce with Fluorescein Isothiocyanate under ultraviolet light. No autofluorescence of the spore walls or cytoplasm was observed.

Normal germination of spores of P. oryzae is shown in Fig. 4, the germ tubes usually arising from the apical cell. Effects of NaCl, Tergitol 4 and Acti-dione are shown in Table 1 and Fig. 5. Germination was inhibited by Acti-dione at all concentrations, although at the lowest level (0.05 mg/ml) germination was reduced only by a factor of two. Many species of fungi, including species of Aspergillus, Penicillium, etc., have been found to tolerate higher concentrations of Acti-dione (2, 3, 4, 5). Any inhibitory properties of this antibiotic would probably be negligible at the 0.05 mg/ml level if added to the medium used in this study for the purposes of inhibiting contaminant molds.

Tergitol 4 (at 0.1% and 0.2%) appeared to be somewhat stimulatory to spore germination, but was severely inhibitory at concentrations

greater than 0.2%. In NaCl, however, germination was enhanced in all concentrations; the germination percent was higher in all cases than that of the control spores (8.3×10^7 viable spores/ml) that were suspended in distilled water.

Effects of the four stains on the viability of spores of P. oryzae are shown in Table I and Fig. 6. Effects of Phloxine B, however, are not shown on the graph (Fig. 6) because it was completely inhibitory to spore germination at all concentrations tested. Rhodamine B also demonstrated inhibition at concentratons greater than 0.5%, although at the 0.1%, 0.2% and 0.5% levels, germination was apparently enhanced, exhibiting a germination percent above that of the control spores (8.3×10^7 viable spores/ml).

Acridine Orange produced varying degrees of enhancement on germination, all concentrations exhibiting percent germination well above the level of the controls. Methylene Blue also demonstrated enhancement of spore germination at all concentrations except 3.0% where germination percent dropped to 6.0×10^7 viable spores/ml, a figure slightly below that of the controls.

Because Acridine Orange stained spores of P. oryzae, visible in white light and exhibiting fluorescence on spore walls under ultraviolet light as well as inducing stimulation of spore germination, this stain might prove to be an excellent one for use in the assay of live or killed spores by direct count and possibly by automation. Methylene Blue might also be considered useful, and, perhaps if the staining solution contained NaCl, its enhancement of spore germination might be increased.

Although NaCl, even at high concentrations, produced no inhibition of spore germination in this study, lesser concentrations in soil and in combination with other soil constituents might demonstrate different properties entirely. Longevity of spores in a soil environment that contained NaCl might be reduced. An investigation to determine the persistence of P. oryzae in various soil types appears warranted.

IV. SUMMARY

Spores of Piricularia oryzae have been stained with a variety of stains. Methylene Blue provided the better staining qualities for use under white light; Acridine Orange and Rhodamine B produced the better fluorescing properties.

Spores of P. oryzae were also suspended in solutions of NaCl, Actidione, Tergitol 4, Rhodamine B, Phloxine B, Acridine Orange and Methylene Blue at varying concentrations. Acti-dione, Tergitol 4 and Phloxine B were found to be severely inhibitory to spore germination; Rhodamine B was somewhat less severe and exhibited some stimulation of germination at concentrations less than 1.0%. Methylene Blue stimulated germination above the control levels at all concentrations except 3.0%. NaCl and Acridine Orange induced stimulation to germination at all concentrations.

Acridine Orange is considered as a possible candidate stain for the counting of spores of P. oryzae directly or by automation. A study of the persistence of P. oryzae in various soil types is suggested.

ACKNOWLEDGEMENT

Thanks are due Specialist E. Haley for technical assistance.

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TABLE I

EFFECTS OF VARIOUS MATERIALS ON VIABILITY OF SPORES OF PIRICULARIA ORYZAE

MATERIALS	CONCENTRATIONS OF MATERIALS USED (%)							
	0.05	0.1	0.2	0.5	1.0	2.0	5.0	10.0
ACTI-DIONE (mg/ml)	3.13*	0.6	ND	0.05	0	0	0	ND
SODIUM CHLORIDE	ND	9.3	ND	17.8	20.0	17.8	17.8	10.0
TERGITOL 4	ND	12.9	9.0	0.2	0.045	0.018	0.03	ND
ACRIDINE ORANGE	ND	12.8	10.0	11.3	13.8	12.5	10.5	ND
RHODAMINE B	ND	12.5	9.3	11.0	0.02	0	0	ND
METHYLENE BLUE	ND	18.5	15.5	12.9	13.9	12.0	6.0	ND
PHLOXINE B	ND	0	0	0	0	0	0	ND

LEGEND: * = Figures are viable colonies (cells) per ml x 10^7

ND = Not done

Average viability of controls = 8.3 x 10^7 cells/ml

EXPLANATION OF FIGURES

Fig. 1 Unstained spores of Piricularia oryzae in Tergitol 4. The one at the top right is aborted in appearance, possibly due to plasmolysis. 1200x.

Fig. 2 Spores of P. oryzae in acridine orange. Spores have their normal physical shape in this stain, but little or no staining in white light. 1200x.

Fig. 3 Spore of P. oryzae in methylene blue. The entire wall has taken up the stain, but details are somewhat hidden. 1200x.

Fig. 4 Spores of P. oryzae with germ tubes arising from the apical cells. 400x.

Fig. 5 Graph demonstrating the effects of Acti-dione, Tergitol 4 and sodium chloride on viability of spores of P. oryzae.

Fig. 6 Graph demonstrating the effects of acridine orange, rhodamine B and methylene blue on the viability of spores of P. oryzae.

FIGURES

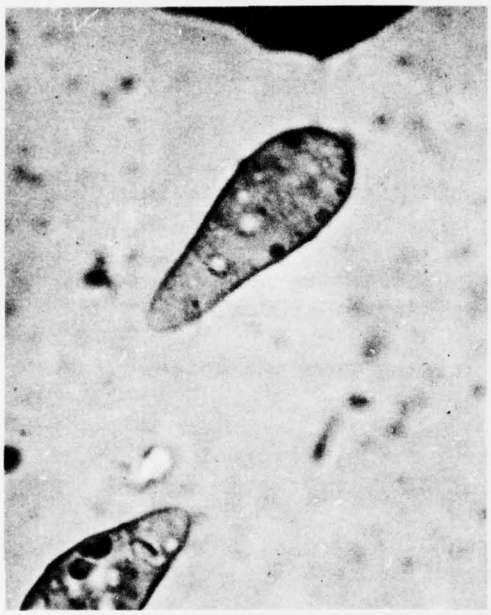
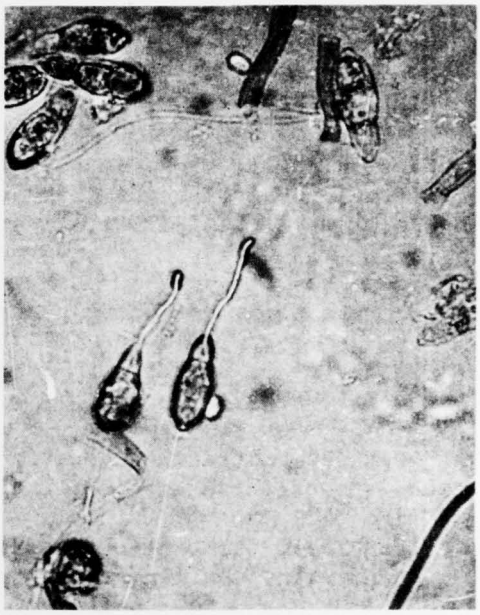


Fig 2



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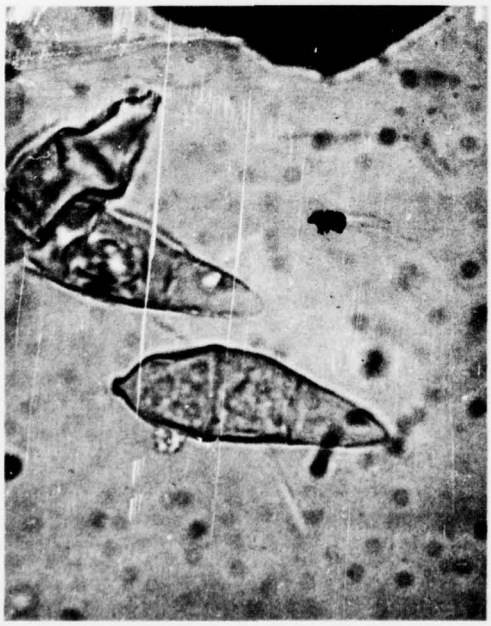


Fig. 1

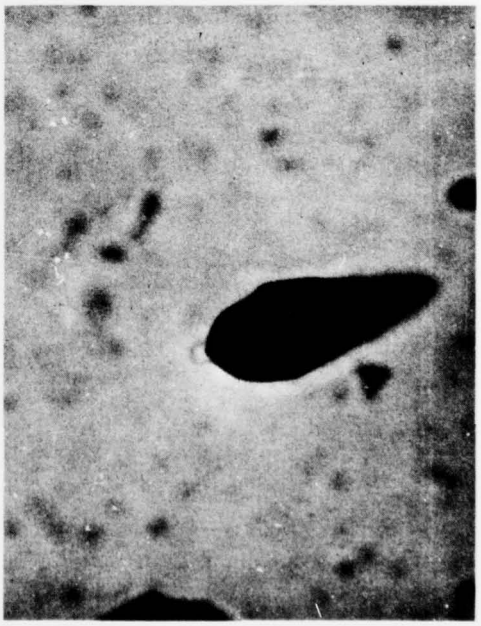
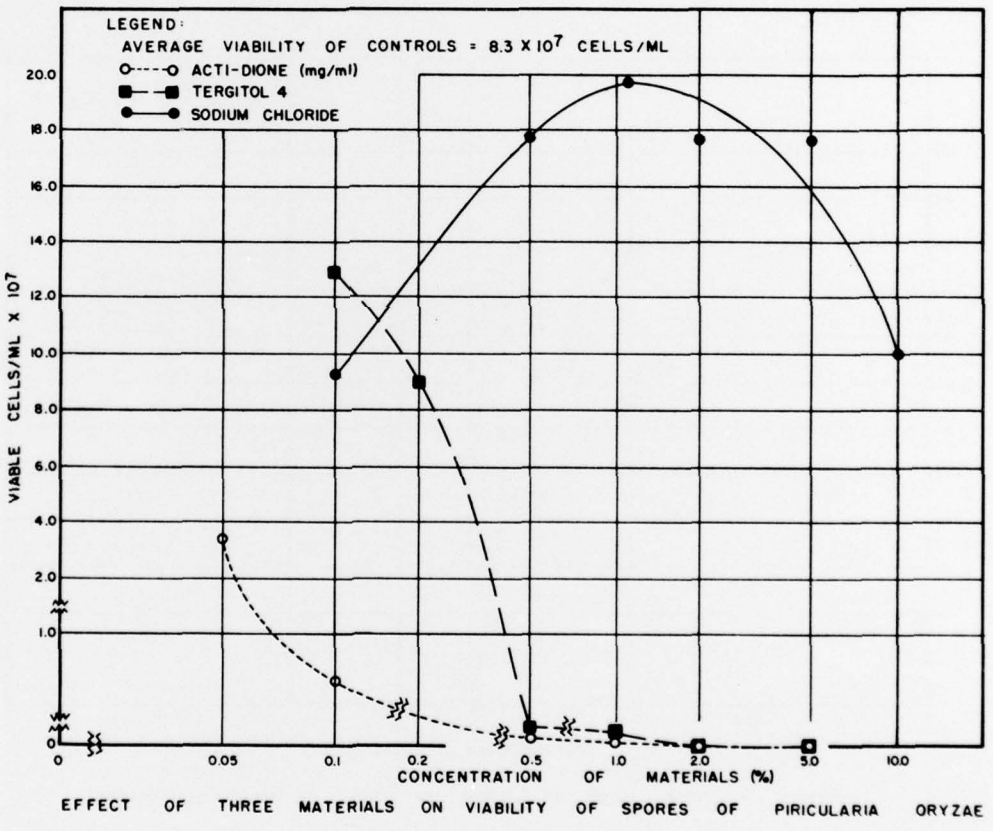


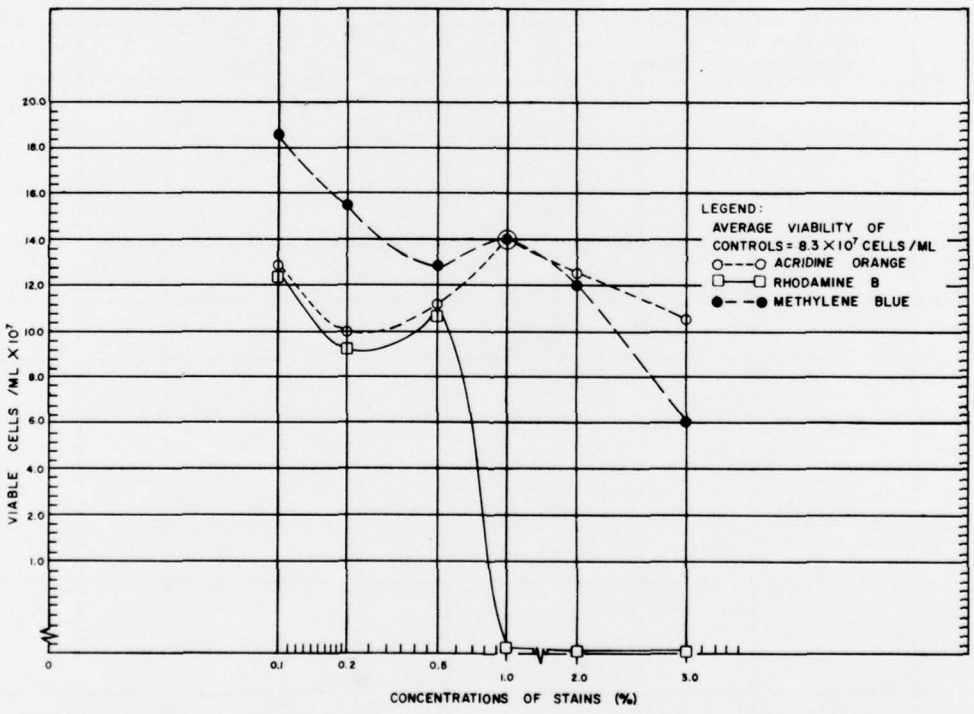
Fig 3

FIGURE 5



EFFECT OF THREE MATERIALS ON VIABILITY OF SPORES OF PIRICULARIA ORYZAE

FIGURE 6



EFFECTS OF THREE STAINS ON VIABILITY OF SPORES OF PIRICULARIA ORYZAE

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Acridine Orange is recommended for staining spores to be counted for direct or automatic counting equipment. A study of persistency of P. oryzae in local soils under ambient conditions is suggested.

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